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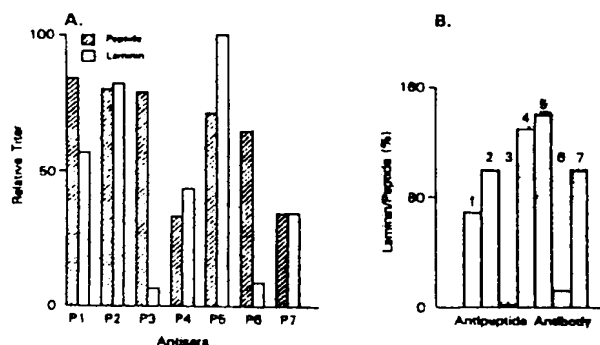
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64 Peptides with laminin activity.

67 Peptides which have laminin-like activity are useful in blocking tumor metastases and are active in cell migration and cell adhesion. All of the subject peptides have the amino acid sequence tyrosine-isoleucine-glycine-serine-arginine.

FIG. 1

Reaction of Peptide Specific Antibodies with Laminin



Description

PEPTIDES WITH LAMININ ACTIVITYFIELD OF THE INVENTION

5 The present invention is directed to peptides having laminin-like activity.

BACKGROUND OF THE INVENTION

10 Laminin (Mr=900,000) is a large glycoprotein specific to basement membranes. Laminin has been shown to promote cell adhesion, cell growth, cell migration, neurite outgrowth, cell differentiation, and to influence the metastatic behavior of tumor cells. Laminin binds to type IV collagen, heparin, gangliosides, and cell surface receptors and promotes the adhesion and growth of various epithelial and tumor cells as well as neurite outgrowth. Laminin is thought to mediate cell-matrix interactions and to be a structural component of all basement membranes binding to collagen IV, heparan sulfate proteoglycan, and nidogen-entactin.

15 The laminin molecule itself has a cross-like shape when examined by microscopy, with three short arms and one long arm. Two small globules can be observed at the end of each short arm, and a larger globule can be observed at the end of the long arm. Current models suggest that laminin contains one A chain (Mr=440,000), one B1 chain (Mr=225,000), and one B2 chain (Mr=205,000), with part of each chain forming a short arm and the rest of the chain projecting down the long arm.

20 Laminin exhibits a number of biological activities, including promoting the attachment, migration, and differentiation of certain cells. Some progress has been made in assigning domains in laminin to its activities. Collagen IV binding is attributed to the globules at the end of the short arms. Cell binding is attributed to the portion of laminin minus the long arm and globules. A site in the long arm of laminin is thought to promote axonal outgrowth. Most of the alpha-helical elements in the laminin molecule have been localized to the portion of the long arm adjacent to the terminal globule. The size of the molecule plus difficulty in separating its chains

25 have impeded further characterization of laminin's structure by conventional chemical approaches. Active domains have been localized in laminin, based on recent progress in cloning the laminin chains. The B1 chain comprises some 1786 amino acids which appear to form at least six contiguous structural domains. Domains I and II are predominantly alpha-helical and probably extend down the long arm. Domains III and V contain homologous repeats rich in cysteine, and could form rather rigid structures adjacent to the globules

30 formed by domains IV and VI. Studies by the present inventors indicate that a sequence of some five to nine amino acids in domain III is at least partly responsible for the cell attachment, chemotactic, and receptor binding activities of laminin. This sequence also has antimetastatic activity with tumor cells.

OBJECTS AND SUMMARY OF THE INVENTION

35 It is an object of the present invention to provide peptides which have useful biological activity. It is a further object of the present invention to provide peptides which have the biological activity of laminin. It is yet a further object of the present invention to provide peptides which have biological activity in the field of cell adhesion and migration and blocking of tumor metastases.

40 It is yet another object of the present invention to provide peptides which block angiogenesis. It is yet another object of the present invention to provide peptides which alter the formation of capillary structures by endothelial cells.

It is yet a further object of the present invention to provide peptides which prevent an excess of blood vessels in tissues due to inflammation or other pathological conditions due to Kaposi sarcoma.

45 Two peptides have been found to have particularly useful properties: a pentapeptide and a nonapeptide, although several other related peptides were nearly as active as the pentapeptide and the nonapeptide.

The pentapeptide of the present invention has the following amino acid sequence:

tyrosine-isoleucine-glycine-serine-arginine.

The nonapeptide of the present invention has the following amino acid sequence:

cysteine-aspartate-proline-glycine-tyrosine-isoleucine-glycine-serine-arginine, i.e., CDPGYIGSR.

50 The entire primary peptide sequence of one of the chains of laminin was determined from cDNA cloning. Using synthetic peptides prepared on a peptide synthesizer, the active domain on the B1 chain responsible for cell attachment and cell migration was identified. Peptides of 20 amino acids and their corresponding antibodies were prepared to each of the seven structural domains. None of these peptides was active, although one of the antibodies blocked cell attachment. Smaller synthetic peptides were prepared to the region around the amino acid sequence specific to this active antibody. A nine amino acid peptide was found

55 to be directly active in cell attachment and cell migration. Various combinations of smaller peptides which authentically matched the protein sequence and/or contained substitutions were tested until the pentapeptide (peptide 5) of the present invention was found to be the most active sequence with the minimal number of amino acids.

DESCRIPTION OF THE DRAWINGS

60 Figure 1A shows the ability of peptide 5 specific antibodies to react with their corresponding peptides and with laminin.

- Figure 1B shows the specificity of the peptide antisera for the B chain of laminin.
 Figure 2 shows that the antibody of peptide 5 inhibits laminin-mediated HT1080 cell attachment.
 Figure 3 shows that peptide 11 can promote HT 1080 cell attachment when coated onto a plastic dish.
 Figure 4 shows that a peptide 11-albumin conjugate can promote HT1080 and CHO cell attachment.
 Figure 5 shows the ability of a peptide 11-albumin conjugate to inhibit attachment to laminin. 5
 Figure 6A shows that peptide 11 elutes a 56 Kd protein, comparable in migration position to the laminin receptor from a laminin affinity column to which NG108-15 cell membrane proteins have been applied.
 Figure 6B shows a Western blot with anti-laminin receptor antibody reacting with the material eluted from the laminin affinity column by peptide 11.
 Figure 7 shows that peptide 11 can inhibit tumor cell invasion in vitro. 10
 Figure 8 shows that YIGSR amide is the smallest active peptide for cell attachment.
 Figure 9 shows that YIGSR amide is the smallest active peptide for cell migration.
 Figure 10 shows that YIGSR amide is the smallest active peptide for tumor cell invasion.
 Figure 11 shows that YIGSR amide is the smallest active peptide for receptor elution.
 Figure 12 shows the effect of YIGSR-amide on capillary endothelial cell tube formation. 15
 Figure 13 shows inhibition of invasion by laminin peptides.

DETAILED DESCRIPTION OF THE INVENTION

The peptides studied were synthesized using a commercially available automated peptide synthesizer (Model 430A, Applied Biosystems, Inc., Foster City, California). Deprotection and release of the peptide from the solid phase support matrix were accomplished by treating the protected peptide on the resin with anhydrous HF containing 10% thioanisole or 10% anisole for one to two hours at 0°C. Following deprotection and release from the resin, the peptides were extracted with either ethyl acetate or diethyl ether. The peptides were then dissolved in 20-50 ml of 10% aqueous acetic acid and filtered to remove the resin. The filtrate was lyophilized to yield white to off-white powders. The composition of all of the peptides was verified by amino acid analysis. Peptides suspected of possessing biological activity were further purified using preparative high pressure liquid chromatography (HPLC) when analytical HPLC indicated that this was necessary. Purity of the pure peptides was verified by HPLC and amino acid analysis. Average yields for all syntheses ranged from 80-90%. The peptides were conjugated to bovine serum albumin for immunization. 20 25

Prior to conjugating a peptide to bovine serum albumin (BSA), the BSA was first derivatized with a nucleophilic spacer, consisting of adipic dihydrazide (ADH). The resulting derivatized BSA (BSA-ADH) was stored as a dry powder at 4°C until used. For the conjugation reaction, the peptide was reacted with an equi-molar amount of bromoacetyl bromide in 5 ml of 0.1M NaHCO₃, and the conjugation reaction was allowed to proceed with magnetic stirring for 18 hours at 25°C. Following the conjugation process, the conjugates were passed through a 2.5 x 30 cm column of Sephadex G-50 in 0.1M NH₄HCO₃. Those fractions containing protein, as judged by their absorbance at 280 nm, were pooled and lyophilized. 30 35

The conjugate was diluted to 1 mg/ml with phosphate buffered saline (PBS) at pH 7.4, and it was mixed with an equal volume of complete Freund's adjuvant for the initial immunization. Rabbits were injected subcutaneously and 14 days later received their second injection. Thereafter, the animals were injected with antigen every eight days for up to eight total injections with antigen. After the first injections, the others were prepared in the same way as the initial antigen, except that incomplete Freund's adjuvant was mixed with it prior to injection. The rabbits were bled, and serum was collected every fourteen days. This serum was checked for antibody titer by enzyme linked immunosorbent assay (ELISA). The ELISA assay was performed in microtiter wells as described by Rennard et al. (Anal Biochem. 104, 205-214, 1980). The antisera were checked for specificity by western immunoblot against laminin. Laminin was extracted and purified from the Engelbreth-Holm-Swarm tumor, which produces a basement membrane matrix, using methods previously described by Timpl et al., J. Biol. Chem. 254, 9933-9939, 1979; Fibronectin was purified from plasma using gelatin-Sepharose. 40 45

HT1080 cells (human fibrosarcoma) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.06% glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin, and 10% fetal calf serum. The HT1080 cells were grown to 80% confluency in Falcon T75 flasks. For adhesion assays, the cells were washed with phosphate buffered saline (PBS), pH 7.3, without Ca or Mg ions, followed by detachment with trypsin 0.025%, EDTA 0.025%. Trypsin was inactivated with medium containing 0.5% BSA. The cells were then pelleted by low speed centrifugation, resuspended in serum-free Eagle's Minimal Essential Medium (EMEM) containing 0.5% BSA (Miles Laboratories), and were then used immediately in the adhesion assay. Cell assays were performed in duplicate and the variability between duplicates was less than 5%. Chinese Hamster Ovary (CHO) cells were maintained in Dulbecco's Modified Eagle's Medium with 0.06% glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin, (v/v from standard stock solution), 1 mM proline, and 10% fetal calf serum. The cells were grown and prepared for the adhesion assay the same way as the HT1080 cells. B16F10, murine melanoma cells, were maintained in DMEM supplemented with 0.06% glutamine, 1% penicillin-streptomycin, 1% non-essential amino acids, and 10% fetal calf serum. Cells were grown to 80% confluency in Falcon T75 flasks. For chemotaxis assays, the cells were washed once in PBS followed by a short trypsinization with 0.025% trypsin, 0.025% EDTA to detach the cells from the dish. The trypsin was inactivated by the addition of DMEM containing 10% fetal calf serum, followed by low speed centrifugation to pellet cells. The pellet was resuspended in serum-free DMEM containing 0.1% BSA. The cells were again 50 55 60 65

pelleted by low speed centrifugation, and resuspended in serum free medium containing 0.1% BSA. These cells were immediately added to the upper compartment of the Boyden chamber.

Cell adhesion was assayed by adding various amounts of laminin and peptide to 35 mm tissue culture dishes and 1 ml of serum-free EMEM containing 0.5% BSA. HT1080 or CHO cells (10^4) were prepared as described above and added to each dish, followed by a one hour incubation at 37°C in 5% CO_2 , 95% air. At the end of this period, plates were gently washed three times with PBS to remove unattached cells. Attached cells were trypsinized with 0.025% trypsin, 0.025% EDTA, and electronically counted. In some cases, bacteriologic petri dishes (35 mm) were coated with either laminin (5 micrograms/dish) or peptide (50 and 100 micrograms/dish) by diluting either in 1 ml of PBS and air drying the dish overnight. Prior to use, coated dishes were rinsed with serum free EMEM containing 0.5% BSA followed by the addition of cells in 1 ml of fresh EMEM with BSA. The remainder of the assay was carried out as described above. In some experiments, varying amounts of peptide were added to laminin-coated dishes (5 micrograms) containing 1 ml of serum-free EMEM with 0.5% BSA, prior to adding cells to see if the peptide could compete with laminin for cell attachment.

Another variation of this assay involved incubating different dilutions of antisera to the peptide albumin conjugates overnight at 4°C with laminin-coated substrates. One hour prior to adding the cells, the unbound antisera were removed, the plates were rinsed with PBS, and 1 ml of fresh serum-free EMEM with 0.5% BSA was added to each plate. The plates were then warmed to 37°C prior to adding the cells. Again the assay was carried out as previously described. The assay measured the ability of antisera to block laminin-mediated cell adhesion.

To measure cell migration, chemotaxis assays were carried out. Polycarbonate filters (8 microgram pore size; Neuroprobe) were coated with type IV collagen (10 micrograms/filter) and placed in a modified Boyden chamber. Cells (B16 F10 melanoma cells) were harvested and prepared as described above, and 3.5×10^5 cells in 0.8 ml were placed in the upper compartment of the Boyden chamber. The lower compartment of the Boyden chamber contained the chemoattractants including various peptides (10-300 micrograms/ml), laminin (20 micrograms/ml), and fibronectin (20 micrograms/ml), in serum-free DMEM with 0.1% BSA (0.2ml). The chambers were then incubated for five hours at 37°C , 5% CO_2 and 95% air. The cells which had attached to the upper side of the filter were mechanically removed. The cells which had migrated to the lower side of the filter were fixed in methanol, and then stained with hematoxylin and eosin. Each sample was assayed in quadruplicate, and the cells in at least 5 microscopic fields per filter were counted.

The ability of the peptides to block migration to albumin was also tested. The preparation in terms of Boyden chamber, filters, and chemoattractants were as described above. The chemoattractants in the lower chamber were fibronectin (20 micrograms) or laminin (20 micrograms/ml). The cells were prepared as described above and placed in the upper compartment of the Boyden chamber with the peptides being tested. The concentration range of the peptides tested was 10 to 300 micrograms/ml.

It has been found that NG108-15 neuroblastoma x glioma cells attach and send out long neuronal processes in the presence of laminin. These cells were cultured to confluence in DMEM containing sodium bicarbonate, 0.06% glutamine, and supplemented with hypoxanthine (1×10^{-4}), aminopterin (1×10^{-7}), thymidine ($1.6 \times 10^{-5}\text{M}$), 1% penicillin-streptomycin, 0.01% gentamicin, and 5% fetal calf serum. These cells were then gently rinsed three times in 0.02M sodium phosphate, pH 7.4, scraped from the dish, and centrifuged at 1000 rpm for five minutes. The cell pellet was then sonicated in 0.01 M Tris-HCl, pH 7.4, containing 1% CHAPS and (3[3-chloromidopropyl] dimethylammonio]-1-propane and 0.002 M phenylmethylsulfonyl fluoride, and extracted for two hours at 4°C . After centrifugation at 10,000 rpm for 20 minutes, the supernatant fluid was applied to a laminin affinity column. Proteins were chromatographed on the laminin affinity columns in a manner analogous to that used to isolate the laminin receptor as described by Lesot et al. (EMBO J 2 861-865, 1983). The sample was circulated on two columns run in parallel and equilibrated in 0.01 M Tris-HCl, pH 7.4, containing 0.1% CHAPS for two hours. After the unbound material was collected, the columns were washed further with the column buffer. Peptides designated as peptide 11 or peptide 12 at 1 mg/ml in the column buffer were added to the columns and allowed to remain in contact with the resin for 10 minutes. The eluted materials were then washed from the column. The starting material, unbound material, and peptide 11 or 12-eluted material were dialyzed against water and lyophilized. Gel electrophoresis in 7.5% polyacrylamide was carried out on aliquots of the samples.

In an analogous study, affinity columns composed of peptide 11 and peptide 12 were prepared and exposed to the NG108-15 cell extracts. The columns were run in a similar manner, and the bound material was eluted with 1M NaCl and 0.1% CHAPS followed by 0.1M glycine, pH 2. The starting material and unbound and bound materials were dialyzed, lyophilized, electrophoresed, and blotted onto nitrocellulose. The nitrocellulose filters were then exposed to anti-67 Kd laminin receptors antibody using established procedures.

Peptides (20 mers) corresponding to each structural domain in the B1 chain of laminin were synthesized as well as conjugated to albumin. The sequences chosen for investigation represent highly hydrophilic regions which were expected to be antigenic, allowing peptide-specific antibodies to be prepared to the conjugates in rabbits. Very specific antibody titers were obtained from immunized animals which were specific to the particular conjugate, and the antibodies could be purified by affinity chromatography on peptide-Sepharose. The antibodies to peptides 1, 2, 4, 5 and 7 showed a good reaction with native laminin in ELISA assays, and these antibodies reacted specifically with the B chain of laminin in Western blots. Antibody to peptides 3 and 6 reacted well with their corresponding peptides but had little or not cross reaction with laminin. The results of these assays are shown in Figure 1A and B.

Each peptide was absorbed to plastic and assayed for its ability to promote the attachment of HT1080 and CHO cells. No significant cell attachment activity was found in any of these peptides. The antisera to the peptides were then also tested for their ability to inhibit epithelial cell attachment to a laminin substrate. Only antibodies to peptide 5 conjugate inhibited cell attachment. The results of this assay are in Figure 2.

Since the corresponding peptide fragment did not promote cell attachment, it was concluded that the actual attachment site occurred in the vicinity of this sequence, and that the active site was blocked by the antibody to peptide 5 for steric reasons. Two peptides, a 9 mer, peptide 11, and a 10 mer, peptide 12, were chosen for synthesis and study based in part on sequence homology to EGF and on their proximity to peptide 5. Peptide 11 was found to stimulate cell attachment in a dose-dependent fashion to the levels obtained with laminin itself. None of the other peptides demonstrated attachment activity when coated on a dish. The results of these assays are shown in Figure 3.

Peptide albumin conjugates were also tested in the cell attachment assays because it was thought that albumin conjugates might increase the availability of the peptide and thus the potency of the peptides in this assay. Peptide 11 albumin conjugate supported the attachment of HT1080 and CHO cells when used at levels of 25 micrograms/ml, while the other peptide conjugates were without activity in this assay. The results of this assay are shown in Figure 4. It was estimated by direct immunoassay that these conjugates represented about 20% peptide, indicating that they show on a molar basis 0.5-1.0% of the activity of laminin itself in cell attachment.

Since it was possible that a completely independent receptor on the cells was binding to peptide 11 rather than the laminin receptor, the ability of these peptides to inhibit attachment to laminin substrates when peptides were added to the coated dishes along with the cells was assessed. Only peptide 11, and none of the others, suppressed the attachment of the cells to laminin. These results indicate that the sequence of amino acids in peptide 11 encompasses the sequence of amino acids involved in attachment to laminin itself. The results of these assay are shown in Figure 5.

Since laminin is known to promote directed cell migration (chemotaxis), the peptides obtained therefrom were tested for chemotactic activity using B16F10 cells, a murine melanoma cell line which is attracted to both laminin and fibronectin. Peptides 1-7 and peptide 12 showed no significant chemotactic activity toward the melanoma cells when placed in the lower compartment of the Boyden chamber, nor ability to inhibit chemotaxis when placed together with the cells in the upper compartment. In contrast, peptide 11, the active portion of which is try-iso-gly-ser-arg, was found to be a chemoattractant for the melanoma cells, showing about 30% of the maximum response observed with optimal levels of laminin. The results are shown in Table I.

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Table I

EFFECT OF SYNTHETIC PEPTIDES
ON CELL MIGRATION

A. Stimulation

Chemoattractant	Cells Migrated	Activity (%)
Laminin	41.6 ± 2.5^a	100
Peptide 11	13.5 ± 1.5	32
Peptide 2	1.8 ± 0.5	4
No additions	3.0 ± 0.7	7

B. Competition

Compound		Cells Migrated	Activity (%)
<u>Lower</u>	<u>Upper</u>		
Laminin	None	4.16 ± 0.7	100
Laminin	Peptide 11	9.7 ± 0.6	23
Laminin	Peptide 2	38.7 ± 0.3	93
Fibronectin	None	16.3 ± 0.6	100
Fibronectin	Peptide 11	12.3 ± 0.8	75
Fibronectin	Peptide 2	15.7 ± 0.4	96%

^aData are presented as \pm SEM

The fact that this was a true chemotactic response was confirmed by the so-called "checker board" assay, wherein the levels of attractants are systematically varied in the upper and lower compartments of the Boyden

chamber.

To establish that the cells recognized similar epitopes on laminin, the ability of the peptides to alter the chemotactic response of the B16 F10 cells to laminin was tested. Only peptide 11 inhibited the chemotactic response of the cells to laminin. Little or no inhibition of chemotaxis to fibronectin was observed, indicating that peptide 11 was showing a specific competition with laminin. These results are shown in Table IB.

Since cells bind to laminin through a specific receptor ($M_r = 67,000$), it was determined whether peptide 11 was able to elute the laminin receptor from a laminin affinity column. A cell extract from NG-108 cells, a murine neuroblastoma cell line which attaches to laminin, was applied to a laminin affinity column and the unbound material was removed with several washes. Subsequently, the column buffer plus peptide 11 (1 mg/ml) was used as eluent, and the proteins in the starting material, unbound material, and peptide eluted material were resolved by electrophoresis. Buffer containing peptide 11 eluted a single major protein ($M_r = 67,000$) as shown in Figure 6, which reacted with authentic antibody to the laminin receptor. Comparable studies with peptide 12 showed no ability to elute the receptor from laminin. The results of this assay are shown in Fig. 6A and 6B.

Peptide 11, Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg, (CDPGYIGSR) and its amide form have been tested in vivo for their ability in vitro and in vivo to inhibit tumor cell invasion and metastases, respectively. In both assays, peptide 11 inhibits tumor cell invasion and metastasis, and the amide form of peptide 11 is even more potent in inhibiting tumor cell invasion and metastasis.

Various peptides from the B1 chain of laminin were tested for their ability to inhibit B16 F10 melanoma cell invasion of a basement membrane in vitro. Only peptide 11 and its amide form were able to inhibit invasion, while peptides 1-7 and 12 were inactive. Peptide 11 was able to inhibit, by approximately 50%, at 50 and 100 micrograms/ml, whereas the peptide 11 amide was more active and able to inhibit invasion by approximately 80% at 100 micrograms/ml. The results of this assay are shown in Fig. 7.

Blind well chemotaxis chambers with 13 mm diameter filters were used for the assay. Polyvinylpyrrolidone-free polycarbonate filters, 8 micron pore size (Nucleopore, California) were coated with basement membrane matrigen (50 micrograms/filter) and placed into Boyden chambers. B16 F10 melanoma cells (3×10^5), suspended in DMEM containing 0.1% BSA, were added to the upper chamber.

Conditioned medium was obtained by incubating fibroblasts for 24 hours in serum-free medium in the presence of ascorbate. This medium was used as a source of chemoattractants and placed in the lower compartment of the Boyden chambers. Assays were conducted at 37°C. in 5% CO₂. At the end of the incubation, the cells on the upper surface of the filter were removed mechanically. The filters were fixed in methanol and were stained with hematoxylin/eosin. Cells from various areas of the lower surface were counted and each assay was performed in triplicate.

Peptide 11 and its amide form were tested in vivo at both 100 micrograms and 1 milligram per animal for their ability to inhibit lung metastases formation. At 100 micrograms, peptide 11 was able to slightly inhibit the formation of lung metastases two weeks after injection into the mice. At 1 milligram, peptide 11 significantly inhibited lung metastases. Peptide 11 amide was more active than peptide 11 at both 100 micrograms and 1 milligram in blocking lung melanoma formation. These data are shown in Tables IIA and IIB.

To test for inhibition of metastases, peptide 11 and peptide 11 amide at 100 micrograms and 1 mg were preincubated with B16 F10 melanoma cells (5×10^5) in a final volume of 0.2 ml. The cells and peptide were injected via the tail vein using standard procedures into C57B1/6 mice. Eight mice were injected for each test concentration. Control mice received only the cells. After two weeks, the mice were sacrificed and the lungs were removed and photographed. Sections were made and the number of metastases was quantified. The results are shown in Tables IIA and IIB, below.

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TABLE IIA

NUMBER OF PULMONARY METASTASES
FROM 5×10^5 MELANOMA CELLS

Two Weeks After Injection into the Mice

	Mean	
	Mean	
	Pulmonary	
	Metastases	% Inhibition
Control	267.8	0
Peptide 11, 100 μ grams	230.0	14.1
Peptide 11, 1 mg	69.5	74.1
Peptide 11 amide, 100 μ grams	151.9	43.3
Peptide 11 amide, 1 mg	4.9	98.4

TABLE IIB

EFFECT OF SYNTHETIC PEPTIDES ON IN VIVO METASTASES

	0	50	100	500 μ g peptide
YIGSR-NH ₂	< 200	70	45	5
cyclic YIGSR	< 200	32	20	3
PDSGR-NH ₂	< 200	65	60	24

Data are expressed as mean number of pulmonary metastases. Eight mice were used for each group.

The peptides were tested for their possible toxic effects on cells. Based on trypan blue exclusion and cell viability, the peptides were not found to have any toxic effects.

Table III shows a series of synthetic peptides having laminin-like activities. The pentapeptide YIGSR, i.e., Tyr-Ile-Gly-Ser-Arg, is the smallest active peptide which is able to promote cell attachment, cell migration and receptor elution, and inhibit tumor cell metastases. The results are shown in Table III and Figures 8, 9, 10 and 11.

TABLE III

Synthetic Peptides Tested for Laminin-like Activities		Relative* Activities			
Laminin		+	+	+	+
1.	C D P G Y I G S R	+	+	+	
2.	C D P G Y I G S R - NH ₂	+	+	+	+
3.	C D P G Y I G S R - 1,2 cyclohexanedione		o		
4.	D P G Y I G S R	+	+	+	
5.	G Y I G S R	+	+	+	
6.	Y I G S R - NH ₂	+	+	+	+
7.	Y I G S R	+	+	+	
8.	Y I G S E		+		
9.	Y G G G R		+		
10.	Y I G S K		+		
11.	I G S R		+		
12.	I G S E		o		
13.	R S G I Y - NH ₂	+	+		
* Attachment of epithelial cells - all were tested					
Migration of tumor cells - all but 3, 4, 8, 9, 10, 11, 12, 13 were tested					
Laminin receptor binding - all but 3 and 13 were tested					
Antimetastatic activity - all but 3, 4, 5, 9, 10, 11, 12 were tested					

As demonstrated above, peptide 11, its amide form, and the active portion tyr-ile-gly-ser-arg, clearly have antimetastatic activity. The peptides can be conjugated to human or homologous albumin, which allows lower amounts to be used, as the albumin conjugate is maintained in the circulation longer than the unconjugated peptide. Additionally, the peptide could be polymerized or cyclized as the polymerized or cyclized peptide would be less likely to be cleared from the circulation and would be effective at a lower dose as an anti-metastatic agent (Table IIB).

The peptides of the present invention can be used as a carrier to target drugs to metastatic tumor cells. Because of this ability to target tumor cells, the peptide can also be conjugated to an anti-cancer agent for therapy.

The peptides of the present invention can be used as a cell-attachment protein to provide substrata to which cells will attach by treating a hydrophobic surface, such as untreated synthetic plastic resin material such as nitrocellulose, or comparable material, with the polypeptide. A similar substratum for cell attachment can be generated by coupling the polypeptide covalently to a solid support, such as glass or a synthetic plastic resin or a long chain polysaccharide, such as agarose, containing a reactive group that can bind the polypeptide. This latter approach can be effected by coupling the peptide to cyanogen bromide-activated agarose beads (sold under the trademark Sepharose by Pharmacia Fine Chemicals, Uppsala, Sweden) sterilizing the beads by autoclaving, and thereafter showing that the peptide coating induces attachment of cells to the beads in a concentration greater than can be obtained by passive absorption.

It has also been found that the peptides of the present invention, i.e., those peptides containing the YIGSR (tyrosine-isoleucine-glycine-serine-arginine) sequence, can alter the formation of capillary structures by endothelial cells, and to inhibit angiogenesis (vascularization). The angiogenesis inhibition was demonstrated in a commonly used assay using chick chorioallantoic membrane.

Human skin endothelial cells plated onto matrigel, a reconstituted basement membrane which is the subject of U.S. Patent Application Serial No. 0161867, filed May 27, 1986, and incorporated herein by reference, rapidly aligned and formed capillary-like structures. The cells showed a very different behavior on plastic- or collagen-coated surfaces, forming a monolayer of single cells. More importantly, the addition of YIGSR amide (tyrosine-isoleucine-glycine-serine-arginine-amide) to the media of cells plated on matrigel, or the inclusion of this peptide (YIGSR amide) within the gel, inhibited the endothelial cells from forming capillary-like structures.

This effect is the subject of Figure 12, which shows the effect of YIGSR-amide on capillary endothelial cell tube formation. In this Figure, A shows endothelial cells which, when plated on reconstituted basement membrane, form tubule-like structures within 18 hours. In B, the formation of the tubules is blocked by 0.5 mg/ml of YIGSR-amide. In C, the cells form a monolayer on type I collagen.

Additionally, the YIGSR amide was tested in the chorioallantoic membrane assay in chick embryos. In these studies, the YIGSR amide placed within the tissue inhibited blood vessels from forming in the region of the peptide.

The peptide YIGSR-amide and a cyclic peptide formed from YIGSR were tested on the invasion of endothelial cells through a reconstituted basement membrane, using the test described in *Cancer Res.* 47: 3239-3245, 1987. In this assay, cells must adhere, degrade, and migrate through the matrix to be considered invasive. As shown in Figure 13, both peptides were potent inhibitors of endothelial cell invasion through basement membrane. This process is also necessary for angiogenesis in vivo. Thus, the YIGSR peptides of the present invention are capable of blocking angiogenesis (neovascularization), indicating that these peptides have a variety of applications in inhibiting growth to preventing an excess of blood vessels in tissues due to inflammation or to other pathological conditions such as Kaposi sarcoma.

The peptides of the present invention can also be used for preparing surfaces for optimal cell culture, derivatization of various prosthetic materials to promote bonding with surrounding tissues, providing for the increased internalization of molecules such as toxins, drugs, hormones, or the like by the enhancement of phagocytosis, and the development of ways of manipulating cellular adhesion mechanisms in diseases such as cancer metastasis and platelet aggregation.

It is expected that such substrata will be useful in cell cultures where it is desirable to ensure proper attachment of the cells. Attachment proteins such as laminin, have been shown to be important for the growth of many types of cells in vitro. Chemically defined media are often supplemented by attachment proteins (cf. Barnes and Sato, *Cell* 22:649-655, 1980). Coating of the culture substratum with the cell-attachment peptide would obviate the use of laminin in the medium, thus providing better defined conditions for the culture, as well as better reproducibility. An example of the commercial use of cell attachment surfaces is the Cytodex particles manufactured by Pharmacia wherein the particles are coated with gelatin, making it possible to grow the same number of adherent cells in a much smaller volume of media than would be possible in dishes. The activity of these beads is, however, dependent upon the use of laminin in the growth medium in most cases. The cell-attachment peptide of the present invention should provide a chemically defined coating for such purposes.

Medical devices can be designed which make use of such substrata to attract cells to the surface in vivo or even to promote the growing of a desired cell type on a particular surface prior to grafting. An example of this is endothelial cell growth on a prosthetic blood vessel or vascular graft, which is generally woven or knitted from polyester fiber, particularly Dacron fiber (a polyethylene terephthalate). Because most types of cells are attracted to laminin and to the peptides of the present invention, the peptides of the present invention are useful in coating a patch graft or the like for aiding wound closure and healing following an accident or surgery. The peptides of the present invention can also be used in coating surfaces of a prosthetic device which is intended to serve as a temporary or semipermanent entry into the body, e.g., into a blood vessel or into the peritoneal cavity, sometimes referred to as a percutaneous device. In such cases, it may be advantageous to couple the peptide to a biological molecule, such as collagen, a glycosaminoglycan, or a proteoglycan.

The peptides of the present invention can be administered in amounts ranging from about 10 micrograms to about 20 milligrams per kilogram of body weight.

The peptides of the present invention may be used in the form of a liquid, such as eye drops or lotions, or a salve or gel which may be applied to promote cell attachment, or in any other convenient form. Accordingly, the peptides may be contained in any pharmaceutically acceptable carrier which is appropriate for the delivery means intended. One manifestation of the cell attachment activity of the peptides of the present invention is their chemotactic activity.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and therefore such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

Claims

1. Peptides characterized in having laminin-like activity and being selected from the group consisting of:
 - (1) tyrosine-isoleucine-glycine-serine-arginine;
 - (2) cysteine-aspartate-proline-glycine-tyrosine-isoleucine-glycine-serine-arginine;
 - (3) aspartate-proline-glycine-tyrosine-isoleucine-glycine-serine-arginine;
 - (4) glycine-tyrosine-isoleucine-glycine-serine-arginine;
 and derivatives thereof selected from the group consisting of amides, conjugates with proteins, cyclized peptides, and polymerized peptides.
2. The peptide of claim 1 which is tyrosine-isoleucine-glycine-serine-arginine.
3. The peptide of claim 1 which is cysteine-aspartate-proline-glycine-tyrosine-isoleucine-glycine-serine-arginine.
4. The peptide of claim 1 which is aspartate-proline-glycine-tyrosine-isoleucine-glycine-serine-arginine.
5. The peptide of claim 1 which is glycine-tyrosine-isoleucine-glycine-serine-arginine.
6. An anti-metastatic agent for metastatic tumor cells, characterized in that it comprises a peptide of claim 1 or its amide form in a suitable carrier for an anti-metastatic agent.
7. A carrier to target metastatic tumor cells characterized in that it comprises a peptide of claim 1 or its amide form.
8. A method for inhibiting the formation of lung metastases, characterized in that it comprises administering to an individual having lung cancer a lung-metastasis-inhibiting effective amount of a peptide of claim 1 or its amide form.
9. A substrate active in promoting epithelial, endothelial or neural cell attachment, characterized in that it comprises a compound selected from the group consisting of a peptide of claim 1, its amide form and its BSA conjugate.
10. A method for promoting increased adhesion of epithelial or endothelial cells to vascular prostheses and other artificial organs, characterized in that it comprises coating the prostheses or organs with a compound selected from the group consisting of a peptide of claim 1 and its amide form.
11. An anti-adhesion factor for laminin-responsive epithelial cells, characterized in that it comprises a compound selected from the group consisting of a peptide of claim 1 and its amide form and its BSA conjugate.
12. An anti-adhesion factor for laminin-responsive epithelial cells, characterized in that it comprises an antibody against BSA or another component conjugated to a peptide of claim 1.
13. A laminin B chain specific antibody, characterized in that it comprises an antibody against the BSA conjugate of a peptide of claim 1.
14. A migration promoting factor for epithelial cells, characterized in that it comprises a compound selected from the group consisting of a peptide of claim 1 and its amide form and its BSA conjugate.
15. A method for promoting the migration of epithelial cells in a wound such as in the cornea and the like, characterized in that it comprises administering to the wound a compound selected from the group consisting of a peptide of claim 1 and its amide form.
16. A migration inhibition factor for laminin responsive epithelial cells, characterized in that it comprises a compound selected from the group consisting of a peptide of claim 1 and its amide form and its BSA conjugate.
17. A method for isolating the laminin cell surface receptor from detergent extracts of cells or of cell membranes bound to a laminin affinity column, characterized in that it comprises adding to said cells or cell membranes a compound selected from the group consisting of a peptide of claim 1 and its amide form.
18. A prosthetic device characterized by having a biologically active surface which exhibits cell attachment activity, said surface having linked thereto a peptide of claim 1.
19. The prosthetic device of claim 18, wherein said surface constitutes a portion of a vascular graft.
20. The prosthetic device of claim 18, wherein said surface is made of a synthetic resin fiber.
21. The prosthetic device of claim 18, wherein said surface comprises a portion of a percutaneous device.
22. A peptide of claim 1 attached to the surface of a synthetic resin fiber.
23. A peptide of claim 1 attached to the surface of a percutaneous device.
24. A surface treated substrate, characterized in that it comprises a peptide of claim 1 attached to the surface of a solid substrate to ensure that cells will attach to said substrate.
25. The surface treated substrate of claim 24, wherein the substrate is selected from the group consisting of glass, synthetic resin, and long-chain polysaccharide.
26. The surface treated substrate of claim 24, wherein the substrate is selected from the group consisting of nitrocellulose and polyester.
27. The surface treated substrate of claim 24, wherein the substrate is agarose.

28. A peptide of claim 1 coupled to collagen.

29. A peptide of claim 1 in the form of a lotion, salve, gel, colloid, or powder.

30. A composition characterized in that it promotes the attachment of cells to a substrate when immobilized on said substrate and comprises a peptide of claim 1.

5 31. A method for inhibiting the formation of capillary-like structures by endothelial cells, characterized in that it comprises administering to a patient an endothelial cell capillary-like structure formation inhibiting effective amount of a peptide of claim 1 or its amide form.

32. A method for blocking blood vessel growth in tissues, characterized in that it comprises administering to a patient a blood vessel growth blocking effective amount of the peptide of claim 1.

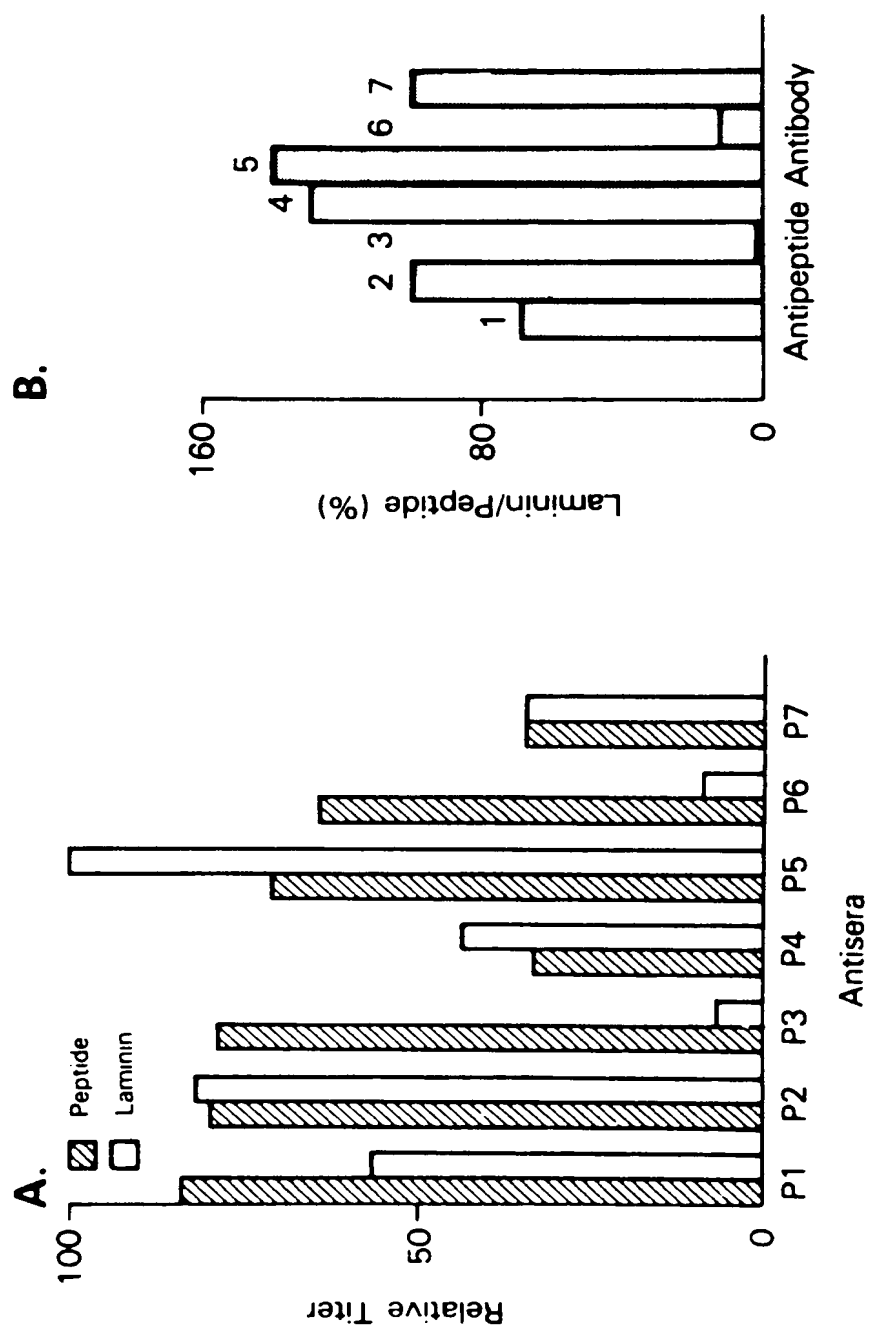
10 33. A method for blocking angiogenesis or new blood vessel formation, characterized in that it comprises administering to a patient an angiogenesis blocking effective amount of a peptide of claim 1 or its amide form.

34. An agent for blocking new blood vessel formation, characterized in that it comprises a new blood vessel formation blocking effective amount of a peptide of claim 1 in a suitable carrier.

15 35. An agent for blocking blood vessel growth in tissues, characterized in that it comprises a blood vessel growth blocking effective amount of a peptide of claim 1 in a suitable carrier.

36. A method for treating Kaposi's sarcoma, characterized in that it comprises administering to a patient a Kaposi's sarcoma treating effective amount of the peptide of claim 1.

FIG. 1

Reaction of Peptide Specific
Antibodies with Laminin

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LAM Pi P1 P2 P3 P4 P5 P6 P7

200K

116K

92K

68K

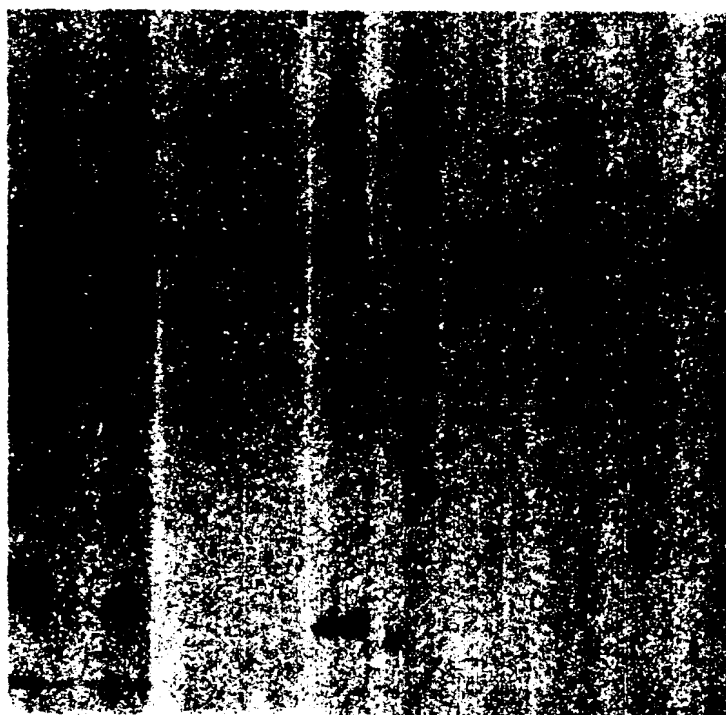


FIG.1B

**Effect of Sequence Specific
Antibodies on HT 1080 Cell
Attachment to Laminin Substrate**

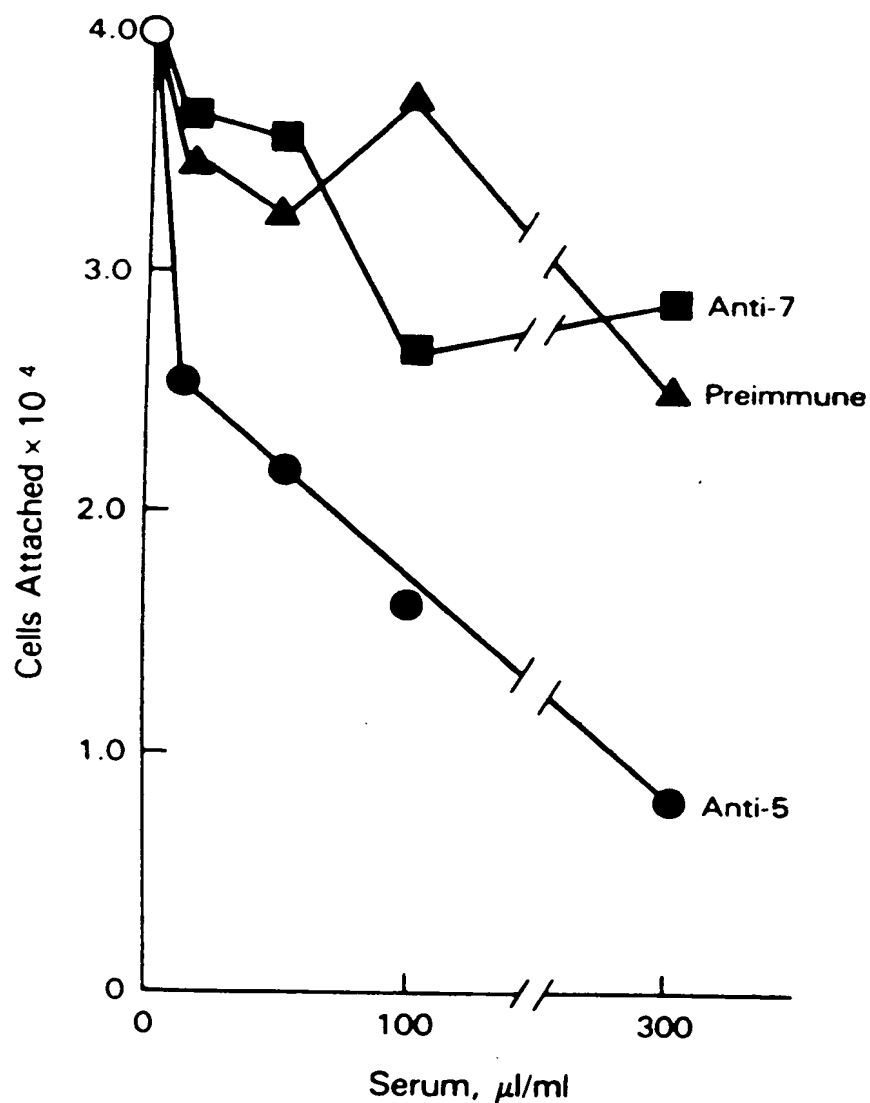


FIG.2

Attachment of HT 1080 Cells to Peptide Fragments

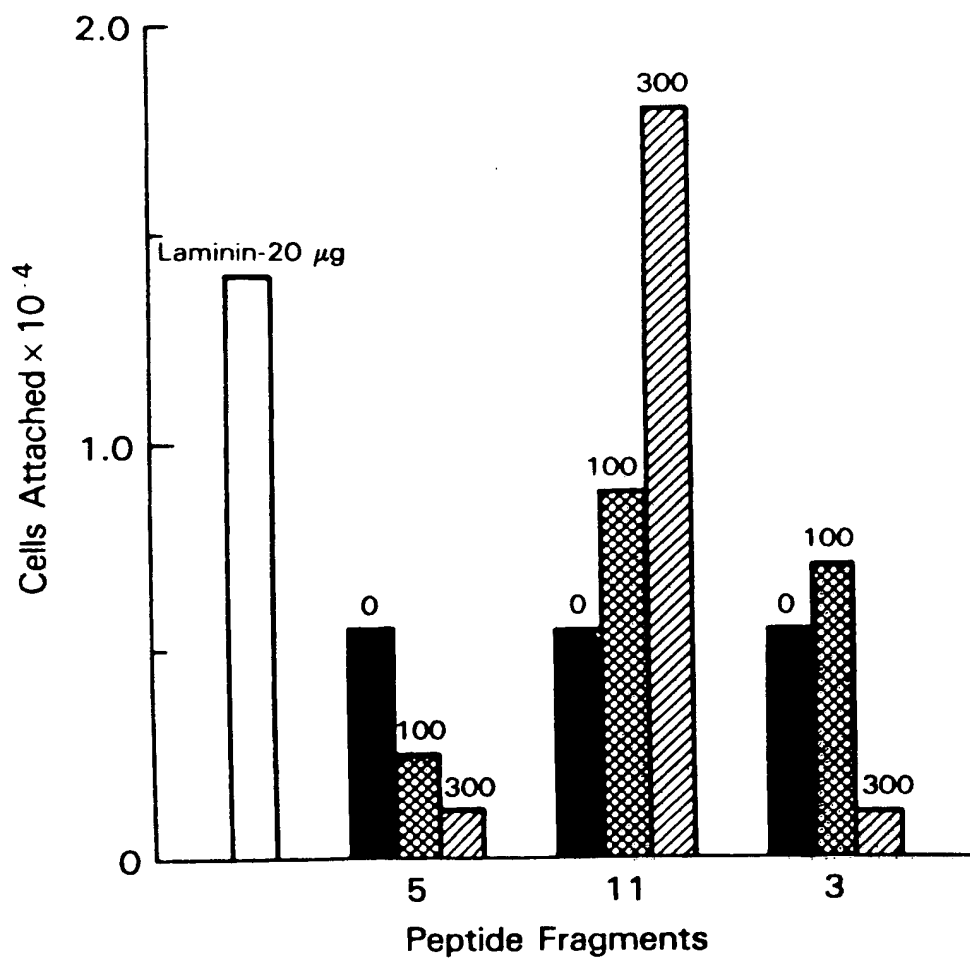
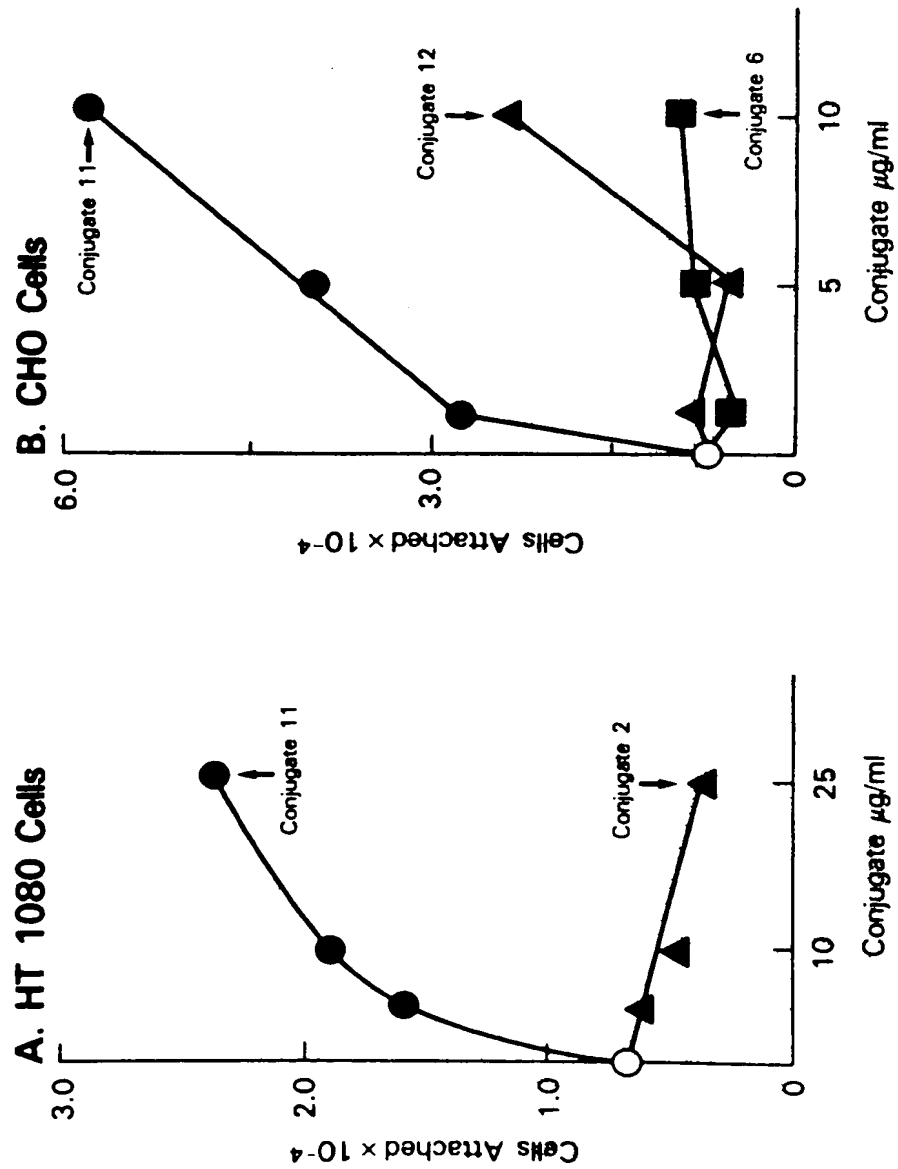


FIG.3

FIG. 4

Attachment of HT 1080
and CHO Cells to Peptide Conjugates

**Inhibition of HT 1080 Cell
Attachment to Laminin Substrate
By Peptide Conjugates**

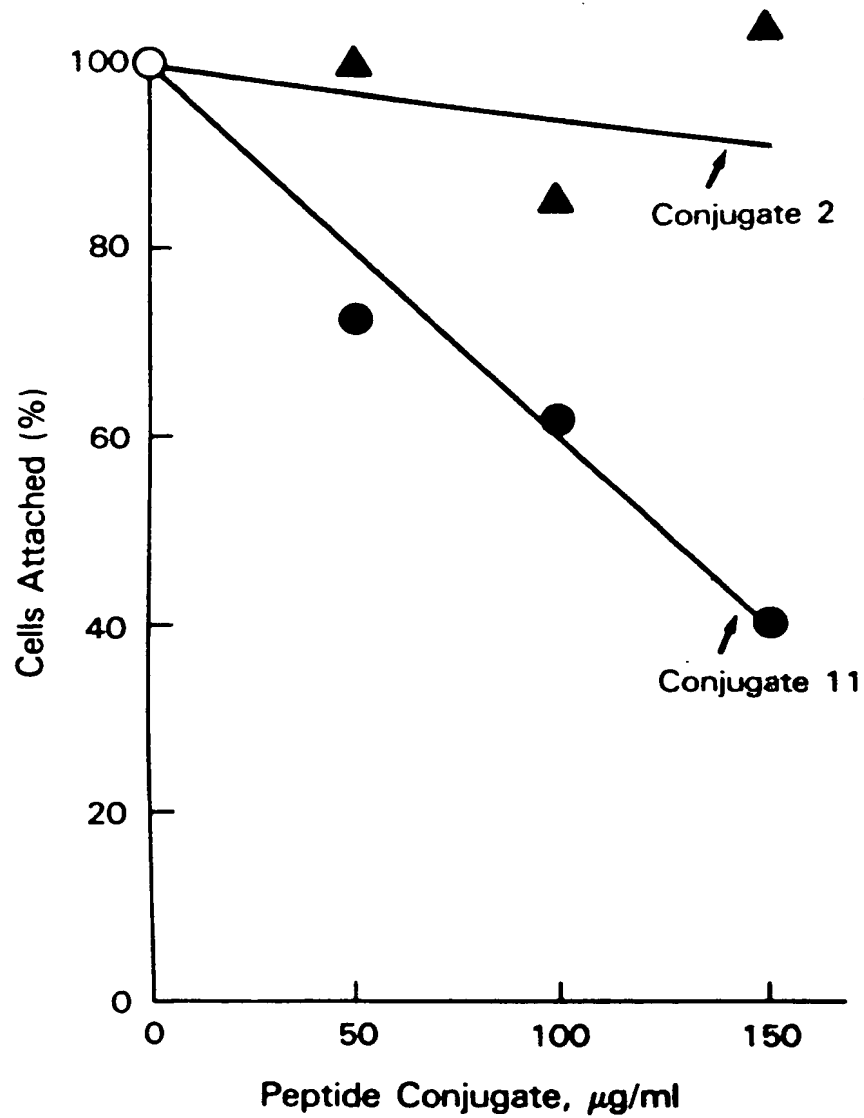


FIG.5

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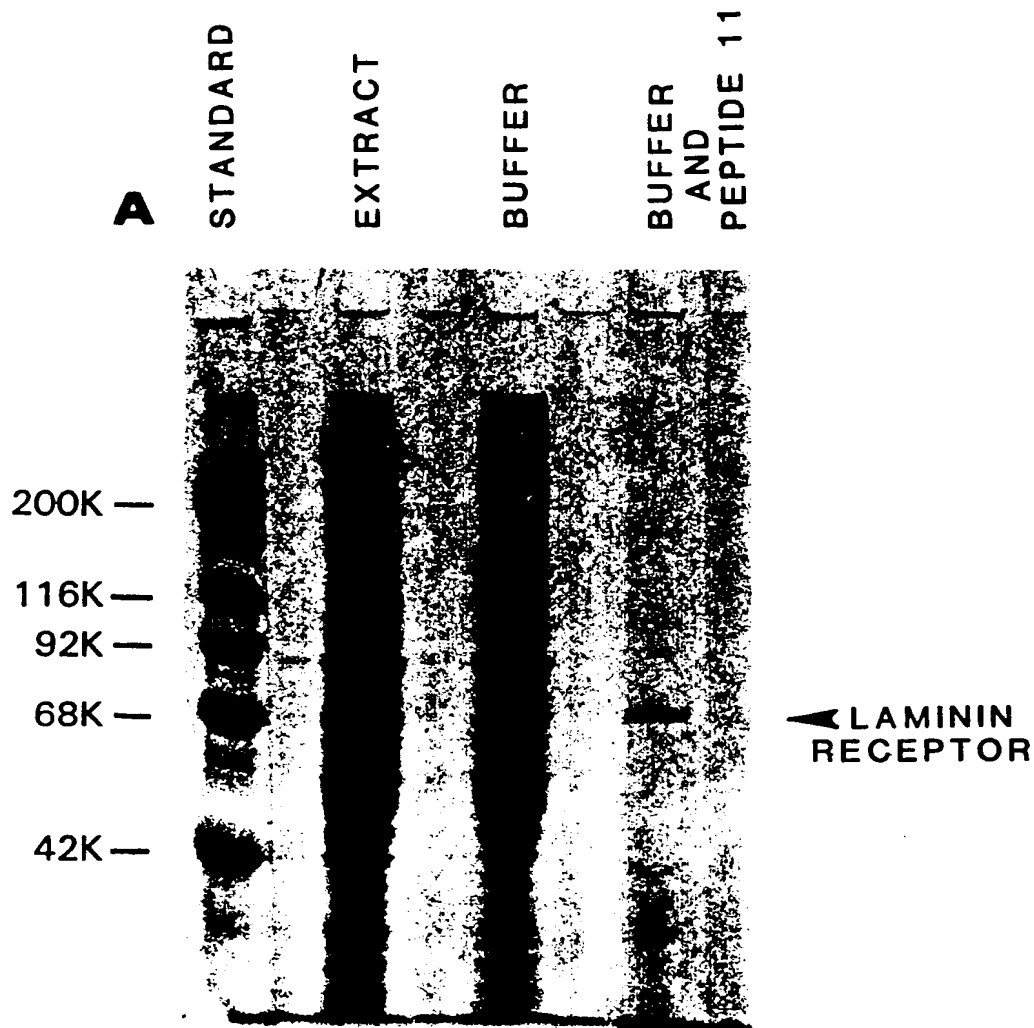


FIG.6A

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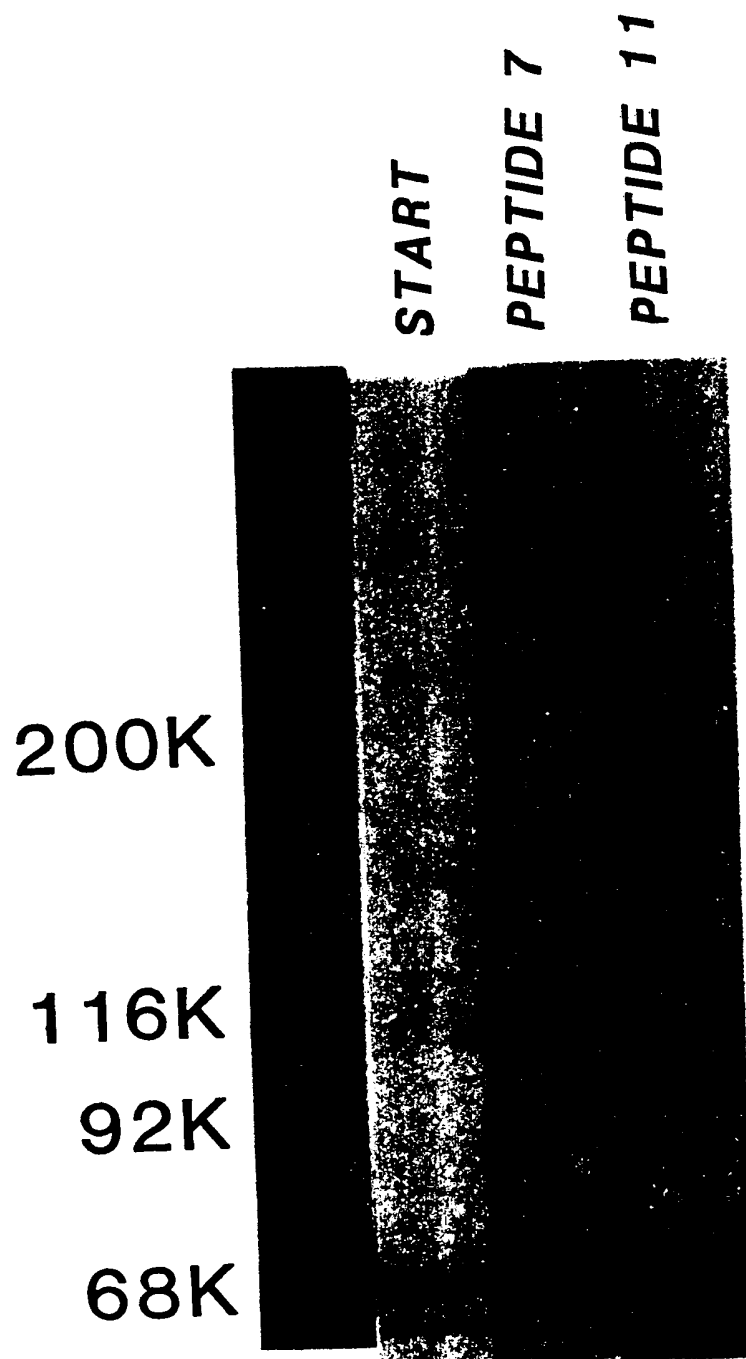


FIG.6B

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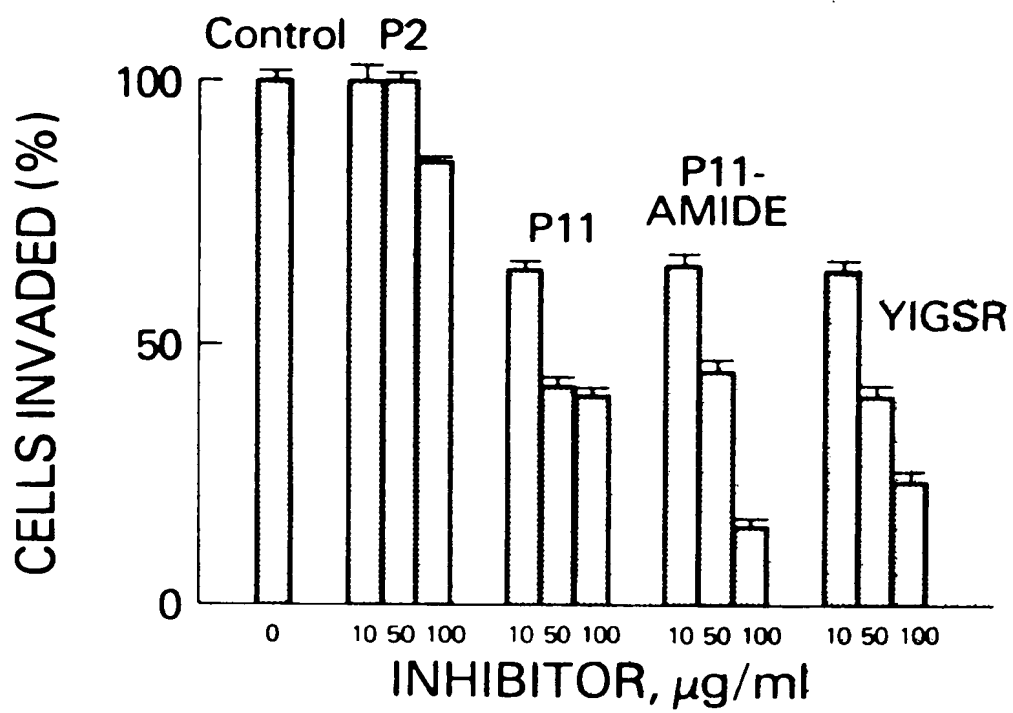


FIG. 7

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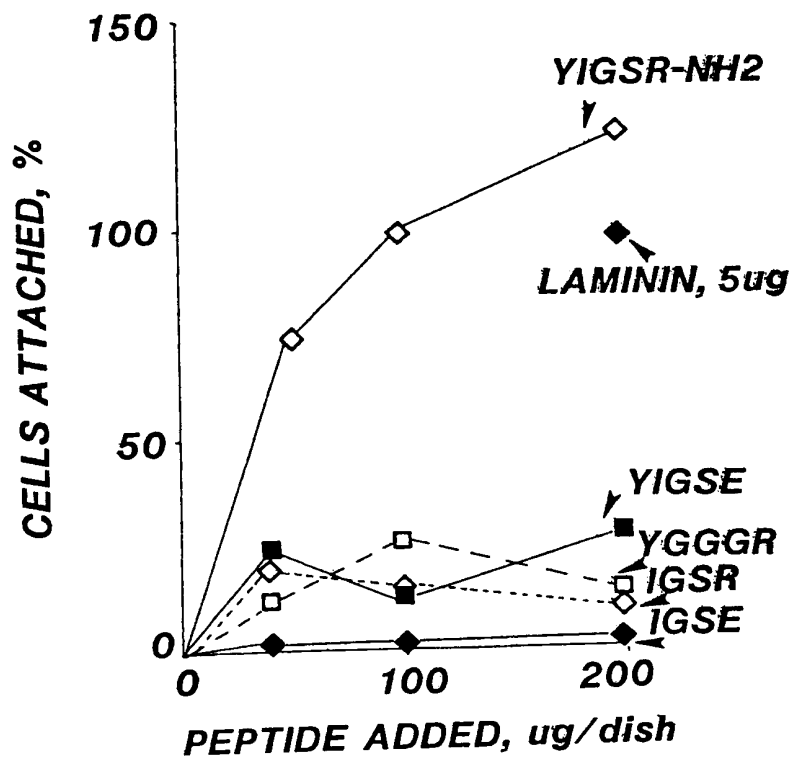


FIG.8

CELL MIGRATION ASSAY

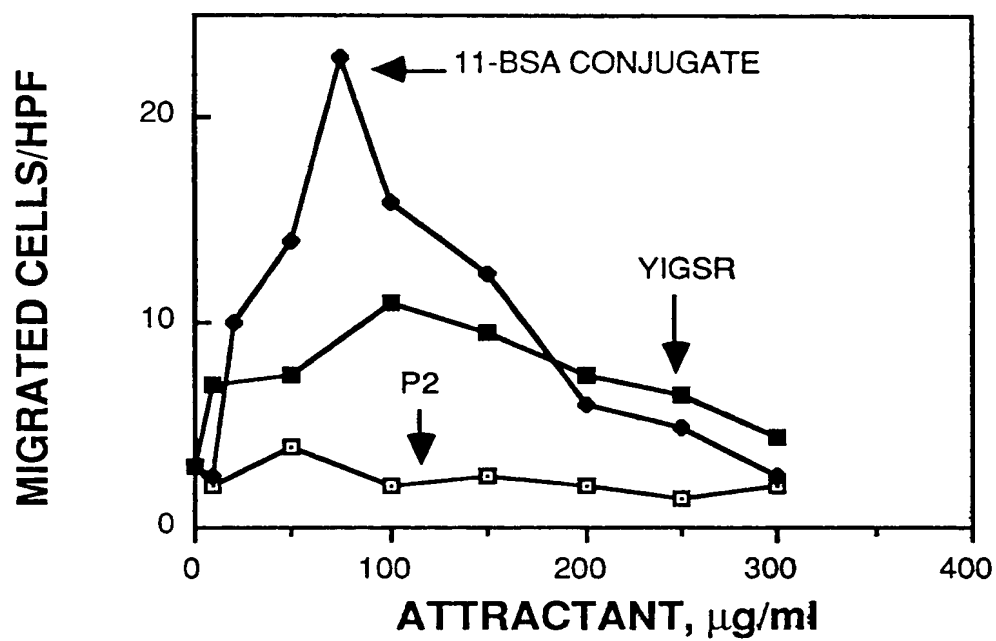


FIG.9

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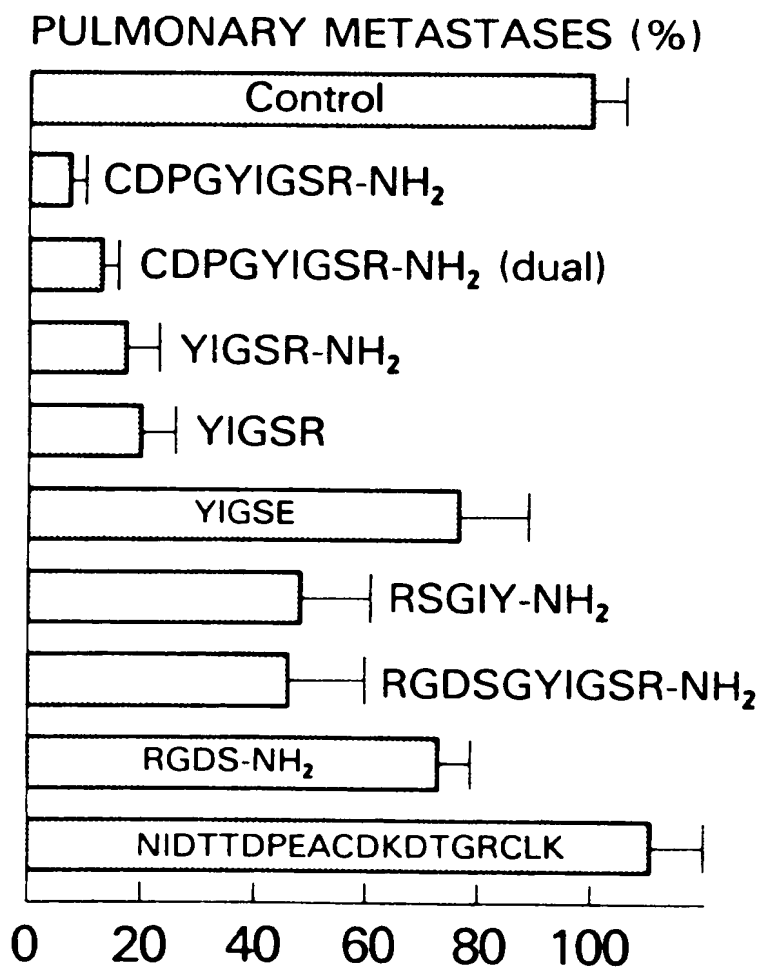


FIG.10

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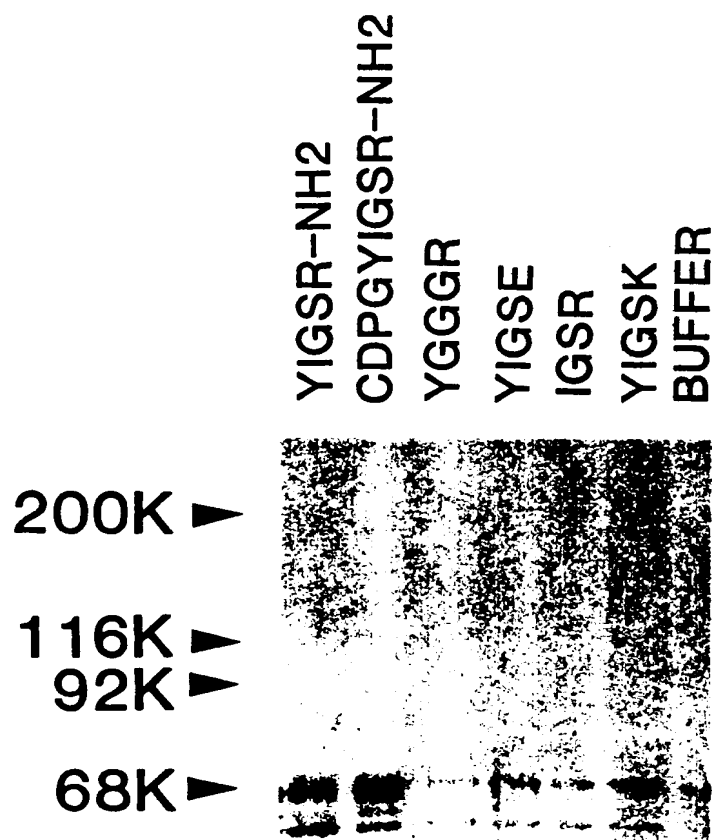


FIG.II

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FIG.12A



FIG.12B



FIG.12C

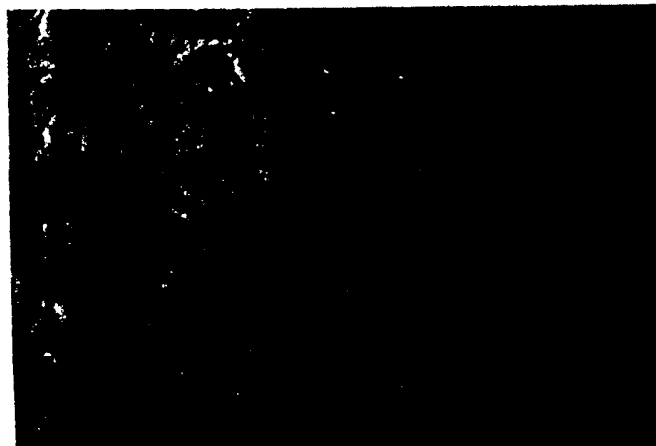
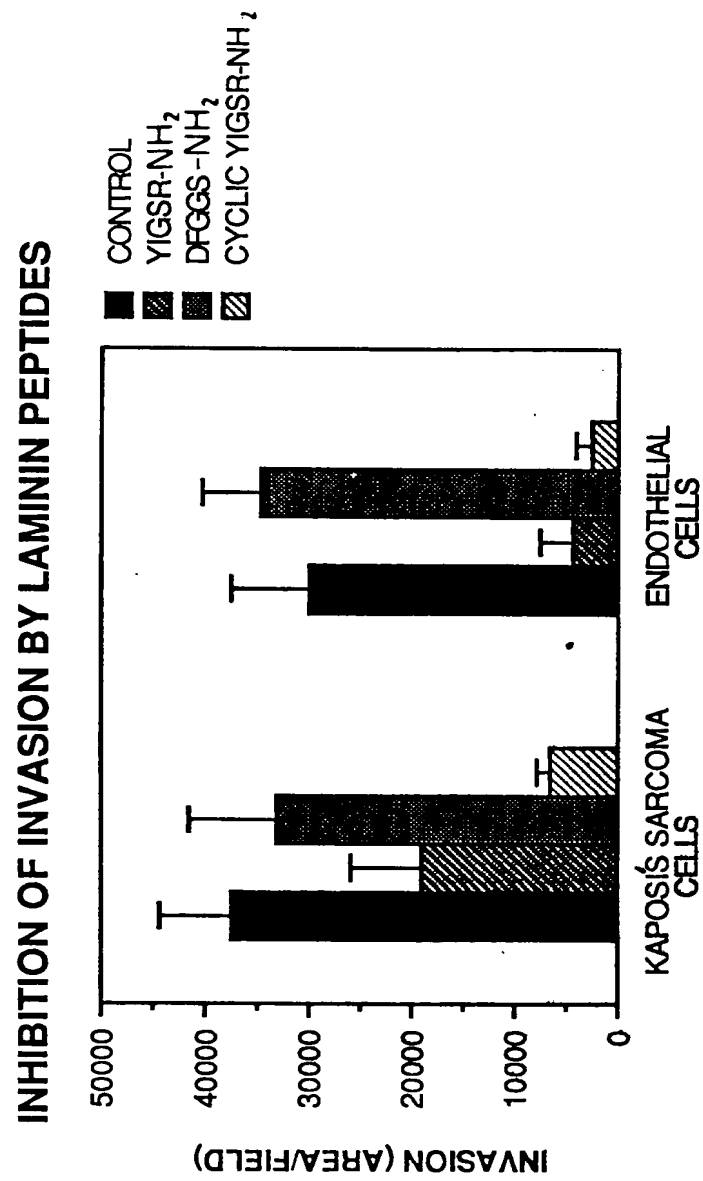


FIG.13



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54 **Peptides with laminin activity.**

57 **Peptides which have laminin-like activity are useful in blocking tumor metastases and are active in cell migration and cell adhesion. All of the subject peptides have the amino acid sequence tyrosine-isoleucine-glycine-serine-arginine.**



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 30 1198

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
A	WO-A-8 400 540 (LA JOLLA CAN. RES. FD.) * Pages 0-3,20-21; figure 1/2 *	1	C 07 K 7/06 A 61 K 37/02 A 61 L 27/00
L	ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 272, no. 1, pages 39-45, 1989, Academic Press, Inc.; H.K. KLEINMAN et al.: "Identification of a second active site in laminin for promotion of cell adhesion and migration and inhibition of in Vivo melanoma lung colonization" * Whole article * (Description of the invention)	1-2,6-36	
P,X	BIOCHEMISTRY, vol. 26, 1987, pages 6896-6900, American Chemical Society; J. GRAF et al.: "A pentapeptide from the laminin B1 chain mediates cell adhesion and binds the 67 000 laminin receptor" * Whole article *	1-2,6-36	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
			C 07 K A 61 K
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 31-01-1990	Examiner RAJIC M.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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